

Site-specific DNA substrates for human excision repair: comparison between deoxyribose and base adducts

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Background: The genetic integrity of living organisms is maintained by a complex network of DNA repair pathways. Nucleotide excision repair (NER) is a versatile process that excises bulky base modifications from DNA. To study the substrate range of this system, we constructed bulky deoxyribose adducts that do not affect the chemistry of the corresponding bases. These novel adducts were incorporated into double-stranded DNA in a site-specific manner and the repair of the modified sites was investigated.

Results: Using restriction enzymes as a probe for DNA modification, we confirmed that the resulting substrates contained the bulky deoxyribose adducts at the expected position. DNA containing these unique adducts did not stimulate DNA repair synthesis when mixed with an

NER-competent human cell extract. Inefficient repair of deoxyribose adducts was confirmed by monitoring the release of single-stranded oligonucleotides during the excision reaction that precedes DNA repair synthesis. As a control, the same human cell extract was able to process a base adduct of comparable size.

Conclusions: Our results indicate that modification of DNA bases rather than disruption of the sugar-phosphate backbone is an important determinant for damage recognition by the human NER system. Specific positions in DNA may thus be modified without eliciting NER responses. This observation suggests new strategies for anti-cancer drug design to generate DNA modifications that are refractory to repair processes.

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Introduction

DNA repair mechanisms are essential for the development and maintenance of life. A few specific DNA lesions may be eliminated by direct reversal, but most types of DNA damage are removed by excision repair mechanisms [1]. In particular, bulky base adducts are processed in prokaryotic and eukaryotic cells by a genetically and biochemically complex pathway, referred to as nucleotide excision repair (NER) [2–4]. This system catalyzes cleavage of the damaged DNA strand on either side of the lesion followed by excision of a single-stranded oligonucleotide containing the lesion. The correct nucleotide sequence is then reestablished by DNA repair synthesis and DNA ligation. The human NER system removes damage from DNA by cleaving the 22nd phosphodiester bond 5' and the 6th phosphodiester bond 3' to the lesion. This dual incision pattern generates single-stranded excision products that are 29 residues in length [5]. *In vitro* repair reactions performed with human cell extracts have confirmed that repair patches induced by this pathway extend over a segment of ~30 nucleotides around the lesion [5,6].

Most eukaryotic cells use NER to remove the principal forms of DNA damage induced by ultraviolet (UV) irradiation [2–4,7]. NER is a highly versatile pathway, however, and has been implicated in the excision of virtually any kind of bulky DNA adduct, including those induced

by the antitumor drug cisplatin, *cis*-diamminedichloroplatinum(II), or the carcinogen *N*-acetoxy-2-acetylaminofluorene [6,8–10]. A common feature of these and other covalent DNA adducts identified as substrates for eukaryotic NER is the modification of the DNA bases. We therefore decided to test whether DNA adducts located on the deoxyribose moiety may also induce an NER response.

Here we report the construction of double-stranded DNA containing single and site-specific deoxyribose adducts. These novel, bulky modifications were obtained by linking a selenophenyl or a pivaloyl group to the C4' position of deoxyribose without affecting the chemistry of the corresponding bases (Fig. 1). When incubated with an NER-proficient human cell extract, DNA containing the selenophenyl or the pivaloyl substitution could not stimulate DNA repair synthesis. Also, the two deoxyribose adducts failed to induce the dual DNA incision pattern that is characteristic of human NER. In parallel control reactions, a bulky guanine adduct formed by *N*-acetoxy-2-acetylaminofluorene stimulated both DNA excision and subsequent DNA repair synthesis.

Results

Preparation of double-stranded M13 DNA containing a site-specific modification

The procedure for the construction of closed circular DNA, containing uniquely located selenophenyl- or

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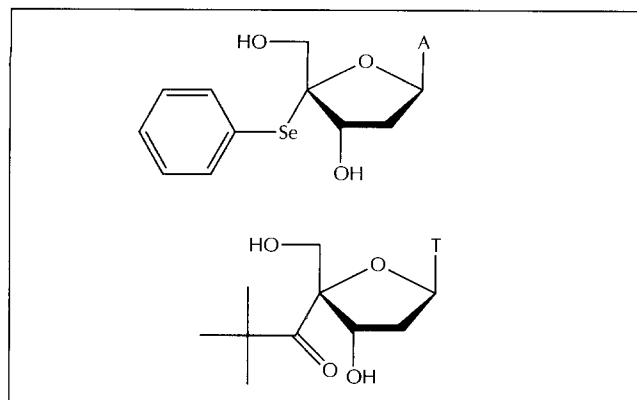


Fig. 1. Structure of the bulky selenophenyl (top) and pivaloyl (bottom) adducts. These novel bulky adducts were covalently bound to the C4' position of a single deoxyribose residue in double-stranded DNA. The chemical structure of the corresponding adenine (A) or thymine (T) base remains unaffected by these deoxyribose modifications.

pivaloyl-deoxyribose adducts, is described in detail in the Materials and methods section. Briefly, the approach taken was to ligate a complementary synthetic oligonucleotide, with the selenophenyl or the pivaloyl adduct located in a central position, into a double-stranded M13-DNA intermediate containing a single-stranded gap (Fig. 2). The ligation products were purified as covalently closed circular duplexes by CsCl gradient centrifugation. M13 DNA with a site-specifically placed acetylaminofluorene (AAF) modification was synthesized by ligating into the gapped intermediate an oligonucleotide containing a single AAF-guanine adduct. A parallel construct with unmodified oligonucleotides was synthesized and isolated using identical methods.

Restriction analysis of covalently closed circular DNA

The site-specific modifications were positioned in the unique *Bst*BI recognition sequence (5'-TTCGAA-3') contained in the M13-DNA derivative used in this study (Fig. 3). We therefore tested the correct location of the AAF, selenophenyl, or pivaloyl adduct by restriction analysis of CsCl-purified substrate with *Bst*BI. Circular, double-stranded M13 DNA, constructed with the unmodified oligonucleotide, was linearized by digestion with *Bst*BI (Fig. 3; lane 4). In contrast, AAF-modified M13 DNA was resistant to digestion by this enzyme (Fig. 3; lane 6). Similarly, *Bst*BI could not cleave selenophenyl-modified (Fig. 3; lane 8) or pivaloyl-modified DNA (data not shown). To rule out non-specific enzyme inhibition, we also determined that all four M13-DNA derivatives were efficiently cleaved by *Pst*I (which recognizes a unique site in M13 DNA proximal to the adduct) and *Alu*NI (which recognizes a unique site at a distance of ~2000 base pairs (bp) from the adduct), yielding linear DNA. The observation that *Bst*BI cannot cleave the modified substrates established that the purified material consisted of homogenous products containing one selenophenyl, pivaloyl, or AAF adduct per M13 DNA molecule at the expected position.

DNA repair synthesis

The purified covalently closed circular M13-DNA molecules were used to determine whether DNA repair components in an NER-proficient human cell extract [6,8] could recognize the DNA adducts. DNA substrates were incubated with soluble HeLa-cell extract in the presence of ATP and all four deoxynucleoside triphosphates, with dCTP radiolabelled (Fig. 4). The HeLa-cell extract contains all factors that are required for damage-specific incision of DNA, excision of the damaged segment, and DNA repair synthesis [6,8,11]. M13 DNA was recovered after reaction with the extract for 3 h and was digested with *Ava*II, *Sma*I, and *Pst*I. This treatment results in the formation of three double-stranded fragments of 37, 330, and 6920 bp (Fig. 4). Previous studies established that *in vitro* repair of bulky modifications, such as the AAF adduct, is strictly dependent on the NER system, which in human cell extracts induces repair patches of ~30 nucleotides around the lesion [5,6]. In our study, quantification of these NER patches is facilitated by the presence

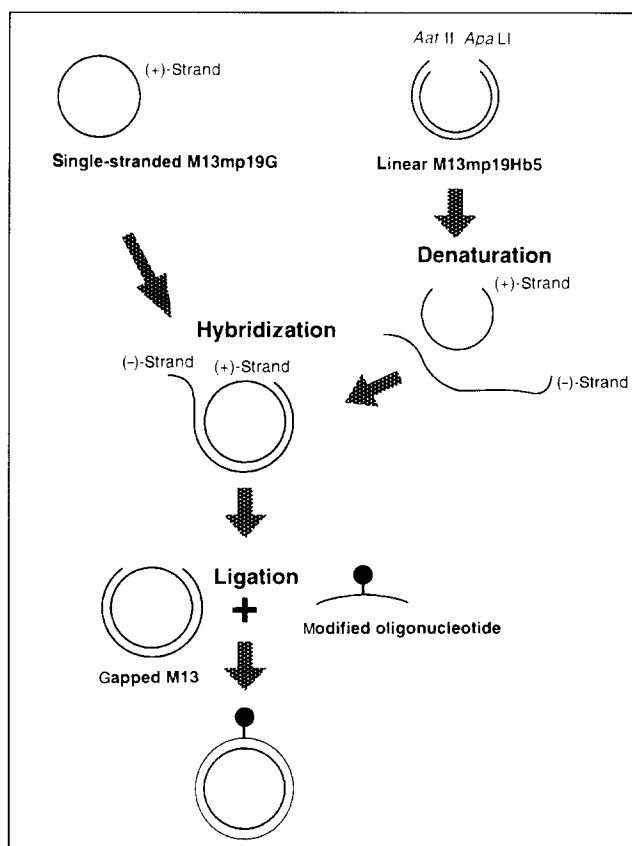


Fig. 2. Schematic representation of the procedure used to construct M13 DNA containing site-specific adducts. The method is based on the synthesis of a gapped M13-DNA intermediate (see Materials and methods for details). A complementary 19-mer (5'-ACCACCCTTCGAACCACAC-3') is ligated into this gapped structure to obtain covalently closed circular M13 DNA. The 19-mer was either undamaged, or contained an AAF base adduct at position G11, a selenophenyl substitution on the deoxyribose moiety at position A12, or a pivaloyl-deoxyribose substitution at position T9. All three modifications fall into the unique *Bst*BI restriction endonuclease cleavage site (5'-TTCGAA-3') of the M13-DNA construct.

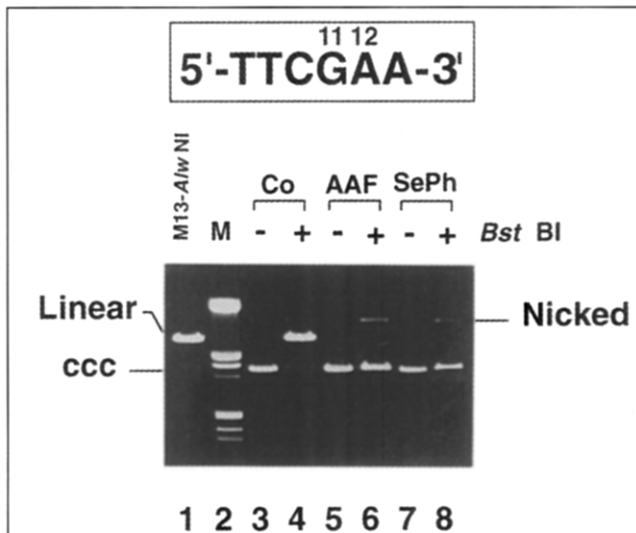


Fig. 3. Modification of the *Bst*BI restriction endonuclease recognition sequence in M13 DNA with an AAF base adduct or a selenophenyl deoxyribose adduct prevents digestion with *Bst*BI. The products of restriction enzyme analysis of M13-DNA derivatives are shown. DNA was subjected to digestion with *Bst*BI and analyzed by agarose gel electrophoresis. The positions of covalently closed circular (ccc), linear, and nicked M13 DNA are indicated. The AAF adduct at position G11 completely inhibited digestion by *Bst*BI (lane 6). Digestion of M13 DNA was also blocked by the site-specific selenophenyl adduct (SePh) situated on the deoxyribose residue of A12 (lane 8). Lane 1: unmodified M13 DNA cleaved by *A/w*NI; lane 2: DNA size marker; lanes 3, 5, and 7: unmodified (Co) or modified DNA (AAF or SePh) before incubation with *Bst*BI; lanes 4, 6, and 8: unmodified or modified M13 DNA incubated in the presence of *Bst*BI. These reactions, performed at 65 °C, produced some non-specific nicking of double-stranded circular DNA.

of the patch within the 37 base pair (bp) *Sma*I–*Pst*I segment of the substrate (see scheme in Fig. 4).

During incubation with HeLa-cell extract, background DNA synthesis occurred randomly, presumably at strand breaks that are either preexisting or induced by non-specific nucleases [6]. As a consequence, reactions with unmodified M13 DNA produced incorporation of radiolabelled nucleotides into all three DNA fragments in proportion to their length (Fig. 5a, lane 1). When the AAF-modified DNA was incubated with cell extract, however, incorporation of radiolabelled nucleotides into the 37-bp *Sma*I–*Pst*I fragment was considerably enhanced (Fig. 5a; lanes 2 and 3, in duplicate). In contrast, when M13 DNA containing the site-directed selenophenyl adduct was incubated with HeLa-cell extract, no increased incorporation of radiolabel in the 37-bp fragment was visible (Fig. 5a; lanes 4–6, in triplicate). Densitometric analysis of three independent experiments demonstrated that the AAF adduct caused a ~70-fold increase in the incorporation of radioactive nucleotides into the 37-bp fragment (Fig. 5b). When the site-specific selenophenyl and pivaloyl adducts were tested, nucleotide incorporation in the 37-bp fragment was not significantly different from that obtained in control reactions with unmodified substrate.

The excision repair assay with selenophenyl-modified DNA was repeated using a different radiolabelled precursor, to ensure that deoxyribose adducts were not repaired by a distinct mechanism, such as base-excision repair, which produces much shorter patches of only one to three nucleotides [12]. In these experiments, [α - 32 P]dCTP was replaced by [α - 32 P]dATP because the selenophenyl group was situated on a deoxyribonucleoside with an adenine base. Incorporation of radiolabelled dAMP into the selenophenyl-containing, 37-bp fragment was again indistinguishable from background levels, excluding the possibility that alternative excision repair pathways for deoxyribose adducts exist. Additional control experiments

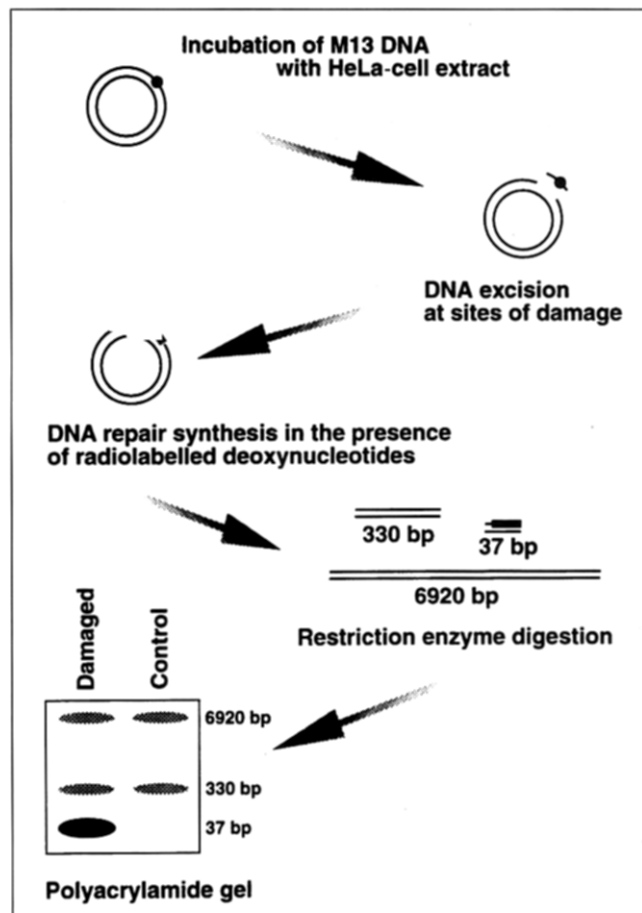


Fig. 4. Schematic representation of the DNA repair synthesis protocol. Substrates containing a site-specific adduct were incubated with HeLa-cell extract, ATP, and all four deoxynucleoside triphosphates, of which dCTP or dATP was radiolabelled. After 3 h at 30 °C, DNA was recovered and digested with *Av*all, *Sma*I, and *Pst*I. This treatment produced a *Sma*I–*Pst*I fragment of 37 bp, expected to contain the DNA repair patch, an *Av*all–*Sma*I fragment of 330 bp located further 5' to the 37-bp fragment, and a long fragment of 6920 bp. The hypothetical electrophoretic analysis illustrates that damage-specific DNA repair synthesis induced by bulky adducts is confined to a region contained within the damaged 37-bp fragment, as the human NER system has been shown to cleave DNA at the 22nd phosphodiester bond 5' and the 6th phosphodiester bond 3' to the lesion [5]. For comparison, the *Sma*I–*Pst*I fragment comprises 23 nucleotides 5' and 13 nucleotides 3' to the AAF site. No difference in non-specific nucleotide incorporation between damaged and undamaged control M13 DNA is expected within the 330-bp and 6920-bp fragments.

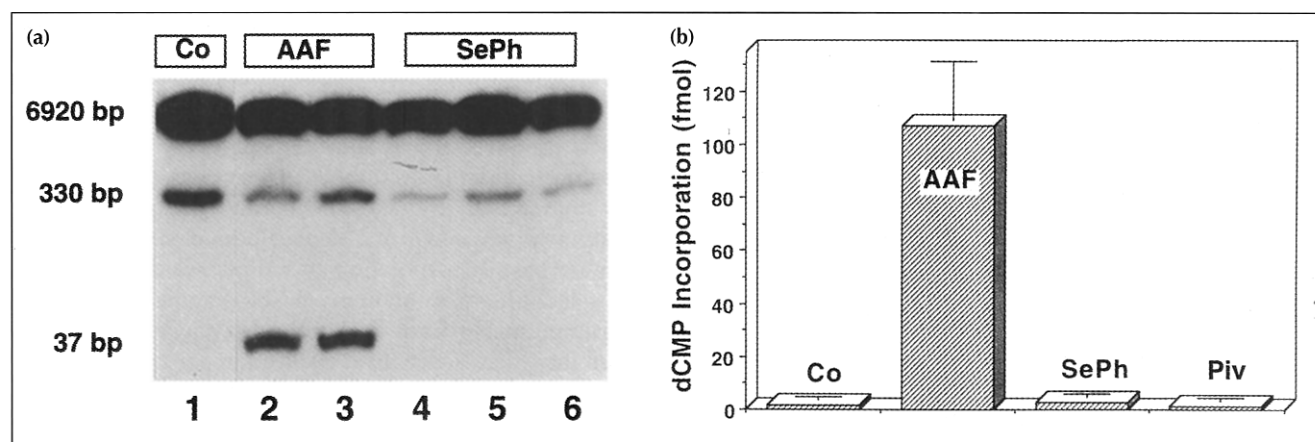


Fig. 5. The deoxyribose adducts do not induce DNA repair synthesis. **(a)** Representative electrophoretic separation of DNA fragments visualized by autoradiography. **(b)** Densitometric quantification of radioactivity incorporated into the 37-bp fragment (mean values of three independent determinations \pm standard deviation) showing AAF-induced repair synthesis into the 37-bp fragment, and indicating that the selenophenyl- and pivaloyl-deoxyribose adducts are largely refractory to excision repair *in vitro*. DNA repair synthesis was expressed as fmol dCMP incorporated into the 37-bp fragment per 250 ng M13 DNA. Co, unmodified M13 DNA; AAF, M13 DNA containing a site-specific AAF-guanine adduct; SePh, M13 DNA with a site-specific selenophenyl-deoxyribose adduct; Piv, M13 DNA containing a site-specific pivaloyl adduct.

were performed in which we determined that DNA repair synthesis at AAF adducts was not affected by the presence of selenophenyl-modified DNA in the same reaction mixture (data not shown), ruling out the possibility that the modified DNA preparations contained a non-specific repair inhibitor.

Dual DNA incision and oligonucleotide excision

Mammalian NER is initiated by cleavage of the damaged strand on either side of the lesion, followed by excision of the lesion as the component of a single-stranded oligonucleotide segment of 29 residues in length [3–5]. To measure this DNA incision and excision activity, we used the *in vitro* assay devised by Huang *et al.* [5]. Site-specifically modified, duplex DNA substrates of 146 bp were constructed by ligating a 19-base oligonucleotide (19-mer) carrying the modification with five other oligonucleotides, as outlined in Figure 6. Prior to ligation, the central 19-mer was labelled with ^{32}P -ATP at its 5' end, such that the resulting 146-bp substrate contained an internal label in the vicinity of the site-specific adduct. This radiolabel was located at the 9th (pivaloyl), 11th (selenophenyl), or 12th (AAF) phosphodiester bond 5' to the site of modification (Fig. 6). The 146-bp substrates were incubated with HeLa-cell extract in the presence of ATP and the four deoxynucleoside triphosphates. After incubation for 45 min, reaction products were detected by denaturing polyacrylamide gel electrophoresis and autoradiography.

Repair reactions containing the AAF-modified substrate yielded excision products that had slightly lower electrophoretic mobility than a 22-mer size marker (Fig. 7a; lane 1). The formation of an oligonucleotide ladder instead of a single band was expected from previous studies and is due to partial degradation of the main 29-mer excision product in the cell-free extract [5]. Whereas AAF adducts were excised with this characteristic oligonucleotide

pattern, indicative of human NER, essentially no excision products were released from DNA containing the site-directed selenophenyl (Fig. 7a; lanes 2 and 5, in duplicate) or pivaloyl adducts (lanes 3 and 6). Similarly, no excision products were released from the control substrate containing no adducts (lane 4), although radioactive bands

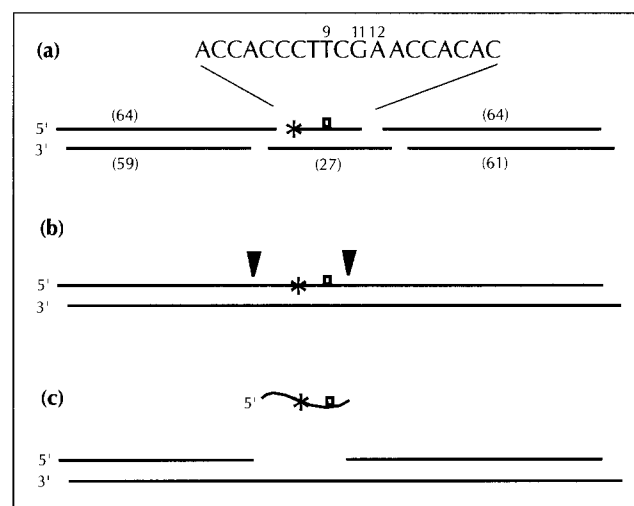
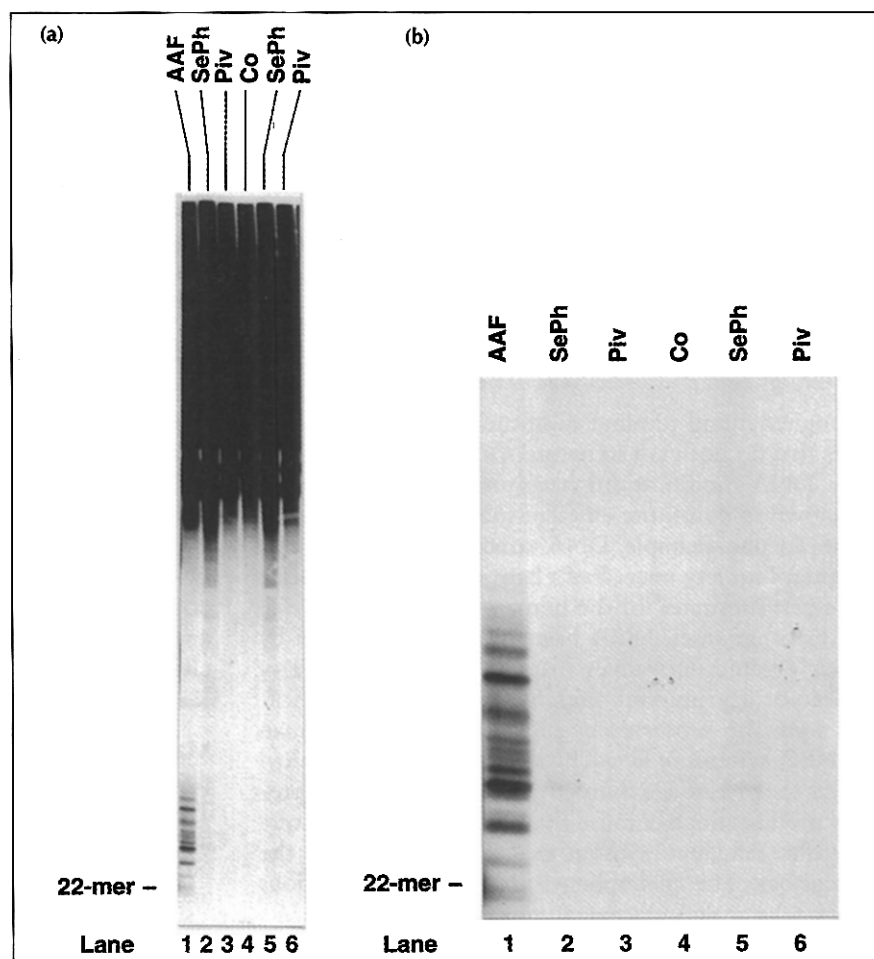


Fig. 6. Schematic representation of the excision assay used in this study. **(a)** Internally labelled double-stranded fragments of 146 bp were assembled from 6 different oligonucleotides. The central 19-mer contained the pivaloyl, AAF, or selenophenyl modification at position T9, G11, or A12, respectively. Control substrate was assembled with unmodified 19-mers. Prior to ligation, the 19-mer was 5' end-labelled with ^{32}P -ATP, and the other five components (lengths in parentheses) were phosphorylated with nonradioactive ATP. **(b)** After ligation, the resulting substrate contained an internal radiolabel near the site-specific modification. The arrow-heads indicate the expected major sites of cleavage by the human NER system. **(c)** Damage-specific dual incision generates radio-labelled products of 29 nucleotides in length. In the cell-free extract, these excision products are partially degraded yielding an oligonucleotide ladder upon electrophoretic separation [5].

Fig. 7. The deoxyribose adducts do not induce DNA excision. Internally labelled substrates containing a site-specific adduct were incubated with HeLa-cell extract, ATP, and all four deoxynucleotides. After 45 min, DNA was recovered and analyzed on denaturing polyacrylamide gels. **(a)** Autoradiograph of the full-length gel with the excision products migrating near the 22-mer size marker. **(b)** Longer exposure of the bottom part of the gel. This autoradiograph was used for quantification of excision products by scanning densitometry. AAF, substrate containing the AAF adduct; SePh and Piv, substrates containing either a selenophenyl- or a pivaloyl-deoxyribose adduct; Co, unmodified substrate.



generated by non-specific nuclease activity were present at comparable levels at the top of the gel. The extent of excision obtained in the reactions with AAF-, selenophenyl-, or pivaloyl-modified substrate was quantified by scanning densitometry of the area of the gel shown in Figure 7b. The corresponding region from the control substrate was taken as background (lane 4). This quantitative comparison indicated that oligonucleotide excision from selenophenyl- or pivaloyl-modified DNA was more than two orders of magnitude lower than that observed with AAF-modified DNA.

Discussion

NER is active on many DNA lesions that differ greatly in their conformation and chemical structure. In fact, this particular repair system processes not only cyclobutane pyrimidine dimers and pyrimidine(6-4)pyrimidone photo-products induced by UV light, but also DNA adducts caused by genotoxic chemicals [2-4,7]. The paradigm of NER in the prokaryote *Escherichia coli* shows that this pathway can process a broad variety of DNA adducts, including highly distortive and helix-destabilizing modifications as well as modifications that stabilize the double-helix, such as those produced by the bulky antibiotic compound anthramycin (reviewed in [13,14]). The hypersensitivity of NER-defective yeast [15] or human cells [16] to anthramycin suggests that the eukaryotic

system possesses a similarly broad substrate range. Recent studies *in vitro* have confirmed that the human NER system recognizes a wide spectrum of DNA adducts, from bulky AAF-, psoralen-, or cisplatin-DNA adducts, to small base modifications such as O⁶-methylguanine or N⁶-methyladenine [8-10].

To gain further insight into the damage recognition mechanism, we compared the activity of human NER on bulky adducts covalently bound either to a base (AAF) or a deoxyribose residue (selenophenyl, pivaloyl). We found that cleavage by *Bst*BI was blocked by the presence of a single base modification or deoxyribose modification near the cleavage site (Fig. 3). An NER-proficient human cell extract, however, was unable to promote detectable levels of DNA repair synthesis in a 37-bp region containing the selenophenyl or pivaloyl adduct (Fig. 5). The sequence environment examined was not generally refractory to NER, as demonstrated by the fact that the same cell extract induced DNA repair synthesis in response to the AAF adduct situated in the immediately adjacent nucleoside position. Mammalian NER is initiated by damage-specific incision 5' and 3' to the lesion, producing oligonucleotide segments of 29 residues in length that are excised from double-stranded DNA [3-5]. As expected, we found that AAF adducts effectively stimulated the release of these excision products. In contrast,

the two deoxyribose adducts were essentially unable to induce oligonucleotide excision (Fig. 7). These studies, performed both at the level of DNA incision/excision and at the level of DNA repair synthesis, indicate that the selenophenyl- and pivaloyl-deoxyribose adducts are not efficiently repaired. Whether a selenophenyl or pivaloyl modification of DNA bases, rather than of the deoxyribose moiety, can be recognized by the NER system has not been tested. An NER response to such base adducts may be inferred, however, from the broad range of base modifications that are susceptible to human NER *in vitro* [10].

Selenophenyl and pivaloyl substitutions are model substrates that do not exist in nature. Other completely synthetic DNA modifications have previously been tested and shown to constitute efficient substrates for the NER system. In one example, DNA containing a site-specific cholesterol moiety instead of a base was found to be one of the best substrates for the human NER system *in vitro* [17]. In other cases, DNA bases have been replaced by various organic derivatives that mimic the presence of apurinic or apyrimidinic sites. Such synthetic modifications were also processed by prokaryotic [18] or human [10] NER systems *in vitro*. The selenophenyl and pivaloyl adducts tested here are distinctly different from these previous modifications because they involve formation of a deoxyribose adduct without replacing or affecting the adjacent base. The selenophenyl and pivaloyl substitutions decrease the melting temperature of duplex DNA (U.S., M.P. and B.G., unpublished data), indicating conformational distortion of the helix. Thus, the low or abolished NER activity in response to these deoxyribose adducts suggests that covalent modification of DNA bases rather than structural alterations of the deoxyribose-phosphate backbone constitutes an important determinant for recognition by the human NER system.

Significance

Using site-specifically modified substrates we show that bulky deoxyribose adducts are processed very inefficiently by the human NER system *in vitro*. This finding contrasts with previous reports demonstrating that NER displays an extremely broad substrate range including virtually every form of base adduct [2-11,14]. It therefore seems that the damage recognition system of NER is primarily sensitive to base lesions, suggesting that bulky substituents may escape this repair process if they are located on certain positions of the sugar moieties in the DNA backbone and do not change the chemistry of the bases.

Several studies have established a correlation between the kinetics with which a particular lesion is removed from DNA *in vivo* and the efficiency with which the same lesion is processed by the human NER system *in vitro* [19-21]. In UV-irradiated human cells, pyrimidine(6-4)pyrimidone

photoproducts are eliminated with a considerably shorter half-life than cyclobutane pyrimidine dimers. A similar relationship was also reported to exist *in vitro*, where the former lesion is a more efficient substrate for the human NER system than the latter [19,20,22]. The absence of detectable excision repair in response to C4' deoxyribose adducts *in vitro* may provide the conceptual basis for a new generation of DNA substitutions that are highly refractory to DNA-repair processes. DNA is the target for a number of clinically useful anticancer drugs, and many new DNA-reactive agents are being tested in clinical trials or developed for potential use as antineoplastic agents [23]. The efficacy of these chemotherapeutic agents probably depends on the number and distribution of unrepaired DNA lesions in cancer cells [23,24]. Our approach, based on deoxyribose chemistry coupled to *in vitro* repair enzymology, may prove useful for the design of new and more effective chemotherapeutic strategies.

Materials and methods

Enzymes

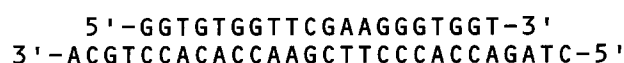
T4 polynucleotide kinase and T4 DNA ligase were purchased from Gibco BRL. Restriction enzymes were purchased from New England Biolabs. Creatine phosphokinase and ribonuclease A were from Boehringer Mannheim. All digestions with restriction enzymes were performed following the manufacturer's instructions.

Modified oligonucleotides

The 19-mer 5'-ACCACCCTTCGAACCACAC-3' was reacted with *N*-acetoxy-2-acetylaminofluorene (NCI Chemical Carcinogen Repository, Rockville, Maryland) to form an AAF adduct at the single guanine (G11) residue [6]. An oligonucleotide with the same sequence but containing a selenophenyl-deoxyribose adduct at position A12 or a pivaloyl-deoxyribose adduct at position T9 was synthesized by the cyanoethyl phosphoramidite method using appropriate building blocks, as described [25]. The oligonucleotides were phosphorylated by incubation with ATP and T4 polynucleotide kinase [26].

Construction of closed circular DNA containing a single modification at a specific position

To produce site-specifically modified M13-DNA molecules we first synthesized a gapped circular intermediate consisting of a circular (+)-strand and a linear (-)-strand (Fig. 2). This gapped intermediate contains a single-stranded region of 19 nucleotides that is complementary to the 19-mer described above. The synthetic DNA duplex:



was ligated into the *Pst*I-*Xba*I site of M13mp19 double-stranded DNA. This ligation generates an M13-DNA derivative, designated M13mp19G, which was used to transform the *E. coli* strain DH5 α F' (Gibco BRL). The resulting single-stranded DNA [27] provides the (+)-strand of the gapped

intermediate. The complementary (–)-strand was obtained by ligation of the duplex DNA:



into M13mp19 double-stranded DNA digested with *Pst*I and *Xba*I, generating a second M13-DNA derivative designated M13mp19Hb5. The insert in this DNA molecule contains the restriction sites for *Aat*II (5'-GACGT'C-3') and *Apa*LI (5'-G'TGCAC-3'). After amplification in the strain DH5 α F', M13mp19Hb5 DNA was purified by CsCl gradient centrifugation [26] and linearized by digestion with *Aat*II and *Apa*LI. The obtained large fragment was then separated by gel-filtration chromatography on a Bio-Gel P-100 column (Bio-Rad). M13mp19G single-stranded DNA (typically 1.2 mg) and the large fragment of M13mp19Hb5 (200 μ g) were coincubated 3 min at 95 °C (denaturation), followed by 15 min at 65 °C and 2 h at room temperature (hybridization), in a volume of 1.3 ml containing 60 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 10 mM EDTA (Fig. 2). The resulting gapped circular DNA (~100 μ g) was purified by chromatography through benzoylated naphthoylated DEAE cellulose (Sigma) and ethanol precipitation as described [28]. Modified 19-mers containing an AAF, selenophenyl, or pivaloyl adduct were ligated into the gapped M13-DNA intermediate in 0.2-ml reactions containing 30 μ g of M13 DNA, 1.0 μ g of phosphorylated oligonucleotides, and 10 U of T4 DNA ligase in 50 mM Tris HCl, pH 7.6, 10 mM MgCl₂, 1 mM ATP, 1 mM dithiothreitol, and 5 % (w/v) polyethylene glycol 8000. After incubation for 4 h at 16 °C, covalently closed duplex DNA was purified by CsCl gradient centrifugation. In a typical preparation, we obtained about 10 μ g of purified final products. Control M13 DNA substrates were constructed by ligating unmodified 19-mers into the gapped DNA.

Assay for DNA repair synthesis

HeLa-cell extracts were prepared by the method of Manley *et al.* [29]. Repair reactions (50 μ l) were slightly modified from Hansson *et al.* [6] and contained HeLa-cell extract (80 μ g of proteins), 250 ng of modified or unmodified M13 DNA, 45 mM HEPES, pH 7.8, 70 mM KCl, 7.4 mM MgCl₂, 0.9 mM dithiothreitol, 0.4 mM EDTA, 3.4 % glycerol, 2 mM ATP, 20 μ M each of dATP, dGTP, and dTTP, 8 μ M dCTP, 2.0 μ Ci [α -³²P]dCTP (3000 Ci/mmol; DuPont NEN), 40 mM phosphocreatine, 2.5 μ g creatine phosphokinase, and 18 μ g bovine serum albumin. Alternatively, incubations were performed in the presence of 8 μ M dATP and [α -³²P]dATP. After 3 h at 30 °C, reactions were stopped by the addition of EDTA to 20 mM. The samples were incubated at 37 °C with ribonuclease A (80 μ g ml⁻¹) for 10 min. SDS (to 0.5 %) and proteinase K (to 190 μ g ml⁻¹) were then added, and the mixtures incubated for a further 45 min at 37 °C. DNA was extracted, digested with *Ava*II, *Sma*I, and *Pst*I, and analyzed by 20 % polyacrylamide gel electrophoresis and autoradiography. This enzymatic digestion produced a *Sma*I–*Pst*I fragment of 37 bp encompassing the site of modification. DNA repair synthesis was quantified by scanning densitometry of the X-ray films on a Molecular Dynamics Computing Densitometer using ImageQuant software. The densitometry was calibrated with radioactive bands that were excised from the dried gel and quantified by Cerenkov counting. DNA repair synthesis in the 37-bp *Sma*I–*Pst*I fragment was expressed as fmol of nucleotides per reaction containing 250 ng of M13 DNA.

Linear DNA substrates

Internally radiolabelled DNA duplexes of 146 bp were obtained by ligating the 19-mer (5'-ACCACCCTTCGAACACAC-3')

with 5 other partially overlapping oligonucleotides (Fig. 6) as previously described [30]. Briefly, the 19-mer (150 pmol) was labelled with [γ -³²]ATP (7000 Ci/mmol; ICN Pharmaceuticals, Inc.) and mixed with 200 pmol each of the other five oligonucleotides, which were phosphorylated with nonradioactive ATP. The mixture was annealed in 40- μ l reactions containing 50 mM Tris HCl, pH 7.9, 100 mM NaCl, 1 mM dithiothreitol, and 10 mM MgCl₂. After heating to 85 °C for 2 min, samples were cooled gradually by incubation at room temperature for 4 h. Ligation was then carried out for 18 h at 16 °C in the presence of 1 mM ATP, 5 U of T4 DNA ligase, and 50 μ g ml⁻¹ bovine serum albumin. The full-length fragment of 146 bp was isolated by electrophoresis in a preparative 6 % polyacrylamide gel under denaturing conditions [26]. Purified DNA was resuspended in 50 μ l of 50 mM Tris HCl, pH 7.9, 100 mM NaCl, and 10 mM MgCl₂, reannealed, and subjected to a second round of purification by 6 % native polyacrylamide gel electrophoresis before incubations with cell extract.

Excision assay

Excision reactions were performed as described [5,10] and contained (in 25 μ l) 35 mM Hepes-KOH, pH 7.9, 60 mM KCl, 40 mM NaCl, 5.6 mM MgCl₂, 2 mM ATP, 20 μ M each of dATP, dCTP, dGTP and dTTP, 0.8 mM dithiothreitol, 0.4 mM EDTA, 3.4 % (v/v) glycerol, 5 μ g bovine serum albumin, 5 fmol radiolabelled DNA substrate, and 50 μ g HeLa cell-free extract. Incubations were carried out at 30 °C for 45 min, and were stopped by the addition of SDS to 0.3 % (w/v) and proteinase K to 0.2 mg ml⁻¹. After proteinase K digestion (15 min at 37 °C) and phenol/chloroform extraction, DNA was resolved by electrophoresis in 15 % polyacrylamide denaturing gels and excision products were visualized by autoradiography. The level of excision was quantified by scanning densitometry of the X-ray films (Molecular Dynamics Computing Densitometer with ImageQuant software).

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